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The ever-expanding *Ly49* gene family: repertoire and signaling

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Summary: The mouse lectin-related Ly49 family and the human killer cell Ig-like receptor (KIR) family represent structurally distinct, yet functionally analogous, class I MHC receptors that are expressed on natural killer cells and some T cells. The functional similarity of these two families has been borne out by the demonstration of identical signal transduction pathways associated with each receptor family. The Ly49 family therefore provides a useful model system to study the role of this class of receptors in the regulation of the immune system. Recent data relating to the Ly49 repertoire in several mouse strains has revealed an additional evolutionary parallel between KIR and Ly49 receptor families. There is now an appreciation of the variation in the number and type of Ly49s expressed in different mouse strains, similar to the previously demonstrated differences in the number of *KIR* genes found in humans. This review summarizes the current members of the *Ly49* gene family, their MHC class I recognition and associated signal transduction pathways.

Natural killer (NK) cells constitute an important facet of the innate immune response against viruses, parasites, intracellular bacteria and tumor cells (1). In addition, NK cells play a role in the regulation of the adaptive immune response. For example, immunoglobulin (Ig) production by B cells in response to antigenic stimulation can be enhanced as a result of NK-cell-derived cytokines (2–5). A role for NK cells in the development of autoimmunity has also been suggested by studies of the murine model of experimental autoimmune encephalomyelitis (EAE) as well as the non-obese diabetic (NOD) mouse (6–8). Therefore, an understanding of the basis of NK-cell control stands to dramatically impact multiple aspects of immunobiology and medicine.

Based on the observation that NK cells preferentially kill targets lacking major histocompatibility complex (MHC) class

I molecules on the cell surface, Ljunggren & Kärre (9) proposed that NK cells recognize and eliminate cells that lack class I (the "missing self" hypothesis). As predicted by this hypothesis, human and mouse NK cells have been found to express receptors for class I MHC that deliver inhibitory signals to NK cells. An unexpected result that was not predicted by the missing self hypothesis is the existence of activating counterparts of inhibitory receptors. The human MHC class I receptors are monomeric type I glycoproteins containing either 2 or 3 extracellular Ig-like domains and they have been referred to as killer cell Ig-like receptors (KIRs). In contrast, the mouse class I MHC receptors are members of the Ly49 family of type II glycoproteins belonging to the C-type lectin superfamily. The Ly49 proteins are expressed as disulfide-linked homodimers, and individual Ly49s are found on subsets of the total NK-cell population. The Ly49 MHC class I receptor gene family is located on chromosome 6 in a region designated as the NK gene complex (10). The Ly49 gene cluster has been extensively studied in the C57BL/6 mouse, and current information suggests that there are 11 active genes (*a–j* and *q*) and five pseudogenes (*k–n* and *v*) in this strain (11–14) (S. K. Anderson, unpublished observations). However, recent work on other mouse strains from several laboratories has indicated that the number of functional Ly49 genes is at least double that found in the C57BL/6 strain, and that at least three of the B6 pseudogenes can be found as active genes in other strains. This review will summarize the currently described *Ly49* genes, their proposed ligands and their signaling capabilities.

Identification of novel Ly49s

Our interest in identifying novel Ly49 family members was sparked by attempts to characterize the Ly49 repertoire in different mouse strains with Ly49-specific monoclonal antibodies. A survey of several mouse strains with anti-Ly49 antibodies revealed considerable heterogeneity in both the level and type of Ly49 proteins expressed (15). The differences observed were not solely a result of class I MHC, since the antibody reactivity of the 129/J strain was very different from the C57BL/6 strain, even though both strains express the same class I haplotype (H2-b). In order to obtain more information about the nature of the Ly49s expressed in different strains, the potential signaling capabilities of the receptors was investigated. Activating Ly49s are readily distinguished from inhibitory Ly49s by immunoblotting with antiphosphotyrosine. When Ly49 immunoprecipitates are resolved on non-reducing SDS-PAGE gels, the inhibitory family members appear as diffuse glycoprotein bands of approximately 100 kDa due to the phosphorylation of the tyrosine residue contained within the immunoreceptor tyrosine-based inhibitory motif (ITIM). Activating Ly49s are revealed by the appearance of a ladder around 32 kDa due to the multiple phosphorylation states of the immunoreceptor tyrosine-based activation motifs (ITAMs) contained within the DAP12 homodimer. Activating Ly49s have been shown to associate with DAP12 via a transmembrane arginine that interacts with a transmembrane aspartic acid residue in the DAP12 molecule (16). We were thus able to identify activating Ly49s that reacted with antibodies previously shown to recognize only inhibitory Ly49s in B6 mice. For example, the 4D11 antibody only recognizes the inhibitory Ly49G receptor in B6; however, an additional activating Ly49 was immunoprecipitated by 4D11 in CBA/J mice. PCR cloning of Ly49 cDNAs from CBA/J mice revealed the existence of a Ly49G-related activator that was homologous to the partially sequenced *Ly49l* gene identified in the B6 genome (17). Expression of the CBA/J-Ly49L protein in 293 cells confirmed that it represented a 4D11-reactive activator. Conversely, inhibitory receptors were identified with an antibody (4E5) that had previously been shown to recognize only the activating Ly49D receptor in B6 mice. Antiphosphotyrosine blotting of 4E5 immunoprecipitates from 129/J NK cells revealed the presence of both activating and inhibitory receptors. Our laboratory has conducted a thorough screening of a 129/J cDNA library, resulting in the isolation of ten distinct Ly49-related cDNAs (18). Three of the 129/J-Ly49 cDNAs (*Ly49o*, *r* and *v*) were shown to produce 4E5-reactive proteins. Ly49O^{129} and Ly49V^{129} are Ly49A^{B6}-related inhibitory receptors, and Ly49R^{129} is a Ly49D^{B6}-related activator. The Ly49s expressed by NOD mice have been studied extensively by Kane's group (7, 8). Alleles of the B6 *Ly49 a*, *d*, *g* and *m* genes are expressed in this strain. NOD mice also express an allele of a *Ly49a*-related activator (*Ly49p*) that was found in 129/J mice. A novel NOD receptor, *Ly49w*, appears to be a chimera containing the first five exons of *Ly49l* and the last two exons of *Ly49g*.

Extensive characterization of the Ly49s expressed by different mouse strains has indicated that the number of intact *Ly49* genes is much larger than previously imagined. It appears that studying the Ly49 repertoire in a single mouse strain can be compared to studying human KIR expression in a single individual. The human *KIR* gene cluster has been shown to vary significantly among individuals, with the number of *KIR* genes in a given haplotype ranging from six to ten (19). Of the several mouse strains that have been investigated, there appears to be a significant difference in the type of *Ly49* genes

expressed, suggesting that the gene content varies among mouse strains. Evidence for highly variable gene content has been provided by McQueen et al. (11), who showed that a single exon probe detected varying numbers of genes in different mouse strains. It is probable that the overall architecture of the *Ly49* gene cluster is conserved between strains, and there are partial or complete deletions of certain genes in individual strains. Unlike the human *KIR* genes, there are several examples of strain-specific inactivation of *Ly49* genes. The *Ly49l* gene produces intact transcripts in CBA, C3H, and BALB/c mice, but no evidence of gene activity has been found in B6 or 129/J mice (8, 17). There are three examples of *Ly49* genes that are present in a given strain, but silenced due to a stop codon in exon 4. The *Ly49m* gene produces a functional transcript in NOD mice, but the B6 allele contains a stop codon at the beginning of exon 4 (8). The B6 *Ly49k* gene has been shown to contain a stop codon at the end of exon 4 (12). Our laboratory has found a *Ly49i*-related gene in 129/J mice that contains a stop codon at the end of exon 4 (S. K. Anderson, unpublished).

The characterization of the *Ly49* gene cluster in C57BL/6 mice has revealed the presence of at least 14 *Ly49* genes (*a–n*). An additional B6 *Ly49* has been recently reported in GenBank (*Ly49q*); however, there is currently no information on the location of the gene for this cDNA. Our laboratory has identified seven Ly49 cDNAs that could potentially represent novel genes in the 129/J strain, and there is currently no report of their presence in the B6 genome. However, there are several large gaps in the B6 map of the *Ly49* cluster that could contain additional genes. In this regard, we have cloned and sequenced an exon 7-containing fragment from B6 genomic DNA that has 98% nucleotide identity (429/436) with the 129 Ly49v cDNA and therefore probably represents the B6 allele of the *Ly49v* gene. Attempts to clone the Ly49v^{B6} cDNA have been unsuccessful, suggesting that it represents a partial gene in B6 mice.

If one takes into account the new Ly49 cDNAs discovered recently, there is now a total of 23 potential *Ly49* genes. *Ly49a, b, c, e, f, g, i, j, o, q, s, t* and *v* are predicted to code for inhibitory receptors, while *Ly49d, h, k, l, m, n, p, r, u* and *w* are predicted to code for activators. The activating receptors have been shown to be more abundant than originally thought, but in the B6 and 129 strains, the number of inhibitory receptors that are expressed is significantly higher than the activating receptors. This is not the case for the NOD mouse, however, since four of the six *Ly49s* described in this strain are activating (*d, m, p* and *w*) (7, 8). Perhaps the balance between activating and inhibitory receptors will have some bearing on the tendency of particular strains to develop autoimmunity. This possibility is supported by the observation that congenic NOD mice containing the B6 NK complex have a reduced incidence of diabetes (20).

It appears that the Ly49 family can be broken down into pairs of activating and inhibitory receptors that have highly homologous (95–97% aa identity) recognition domains. Examples of homologous receptor pairs include Ly49O/Ly49D, Ly49I/Ly49U, Ly49C/Ly49H and Ly49G/Ly49W. This may be a reflection of the mechanism by which the *Ly49* gene family has expanded. Duplication of an inhibitory receptor was probably followed by recombination with an activating receptor. The investigation of the nature of the *Ly49* gene family has come to a point where the determination of the genomic sequence of the *Ly49* gene cluster and the number of expressed genes in several mouse strains will be crucial to obtaining a complete understanding of the evolution and function of these genes. Comparative analysis of the promoter regions of various family members may yield information on regions that are critical for either the cell-specific or the temporal regulation of family members.

MHC class I binding of Ly49 proteins

The MHC class I-binding characteristics have been studied for the majority of known Ly49s, either in cell-based assays or by class I tetramer staining (7, 8, 18, 21–26). *Fig. 1* shows a listing of all the currently defined Ly49 proteins, the predicted class I MHC ligands and antibody reactivities. The identification of a large number of Ly49s capable of binding to the D^d molecule has made it possible to generate a consensus Ly49 sequence associated with D^d binding. *Fig. 2* shows the 8/8 consensus sequence generated with the Ly49A, D, G, O, P, R, V and W sequences. The consensus sequence contains almost all of the residues identified as interacting in the Ly49A-D^d complex by Tormo et al. (27). The putative contact residues that are not conserved in all eight family members represent differences that are found in the Ly49G and W proteins, and may account for the decreased binding of these receptors to D^d relative to Ly49A. The only D^d-binding Ly49 that does not fit this consensus is Ly49C, which has been shown to bind a broad range of class I molecules. In fact, almost every proposed contact residue is different in Ly49C, and the five residues that are conserved are found in every Ly49 family member. A consensus can also be generated for D^b-binding Ly49s, and in this case, the only Ly49 that does not fit is the promiscuous Ly49V molecule. This suggests that the Ly49s capable of binding to multiple class I haplotypes

Ly-49	Putative Ligand	Monoclonal Antibody
A	D^b, D^d, D^k	A1, YE1/48
B	?	
C	H-2^b, K^d, D^d, D^k	5E6, 1F8, 4L011, 14B11
D	D^d, D^r, D^{sp}	4E5, 12A8
E	?	
F	?	14B11
G	D^d, L^d	4D11, Cwy-3
H	D^b	1F8, 14B11
I	K^d	5E6, 1F8, 8H7, 14B11
J	?	8H7
L	H-2^k	4D11
M	?	
O	D^b, D^d, D^k, L^d	4E5
P	D^d	A1, YE1/48
Q	?	
R	D^d, D^k, L^d	4E5, 12A8
S	?	
T	?	YE1/48, 4D11
U	?	1F8
V	H-2^b, H-2^d, H-2^k	A1, YE1/48, 4E5
W	H-2^d, H-2^k	4D11, Cwy-3

Fig. 1. Characteristics of known Ly49 proteins. A complete list of the predicted Ly49 proteins that are currently described is shown, with the exception of the *Ly49k* and *n* gene products, for which no complete protein coding sequence is known. Ly49A–J and Q are based on information derived from the C57BL/6 proteins; Ly49L is from CBA/J; Ly49O, P and R–V are from 129/J; and Ly49M, P and W are from NOD mice. Inhibitory Ly49s are listed in red type and activating receptors are listed in green. The predicted class I MHC ligands of each receptor is listed, based on functional assays and binding of soluble class I MHC tetramers. The Ly49 proteins that have been shown to bind D^d are indicated by the shaded boxes. The monoclonal antibodies known to react with each receptor are listed.

may use contact residues outside of those identified for the Ly49A-D^d interaction. Mutational analysis of the Ly49C protein by Lian et al. has demonstrated the importance of two C-terminal isoleucine residues in the recognition of class I MHC (28). In a cell–cell binding assay, Ly49C binds to cells expressing D^d, D^b, K^b, K^k, H-2^b and H-2^s class I molecules, whereas the highly related Ly49I receptor does not. Substitution of isoleucine 226 of Ly49C with the corresponding Ly49I residue (threonine) resulted in the loss of MHC binding. Replacement of isoleucine 247 with threonine only affected the ability of the mutated Ly49C to bind D^d and H-2^s-expressing cells. This result is consistent with the polymorphic region adjacent to isoleucine 247 playing a role in allelic specificity as indicated by the crystal structure of Ly49A (27). Whether or not these two residues actually contact class I and contribute to the broad specificity of Ly49C can only be resolved by the determining the structure of Ly49C bound to class I MHC.

Signaling by inhibitory Ly49s

As our understanding of the genetics of the Ly49 repertoire expands, the potential impact of this family of receptors is magnified. Therefore, in an effort to understand the biochemical functions and, thereby, the physiological role of these receptor systems, several groups have been characterizing their signal transduction pathways. These studies, like those of the human Ly49 counterparts, the KIRs, initially followed in the footsteps of individuals studying inhibitory pathways activated by the Fc receptor of B cells, FcγRIIb. Early work on this receptor showed that co-engagement with the B-cell receptor (BCR) inhibited immunoglobulin production (29, 30). Mutagenesis of FcγRIIb demonstrated that a tyrosine-based motif within its cytoplasmic tail was required for inhibitory activity (31). Subsequent studies showed that phosphorylation of the tyrosine residue within this motif mediated the recruitment of cytosolic protein tyrosine phosphatases (32).

Although it has recently been demonstrated that most of the inhibitory activity of FcγRIIb is mediated via lipid phosphatases, not protein phosphatases, this early work led to the description of the ITIM (32). The motif was defined as containing a tyrosine in configuration for phosphorylation, fol-

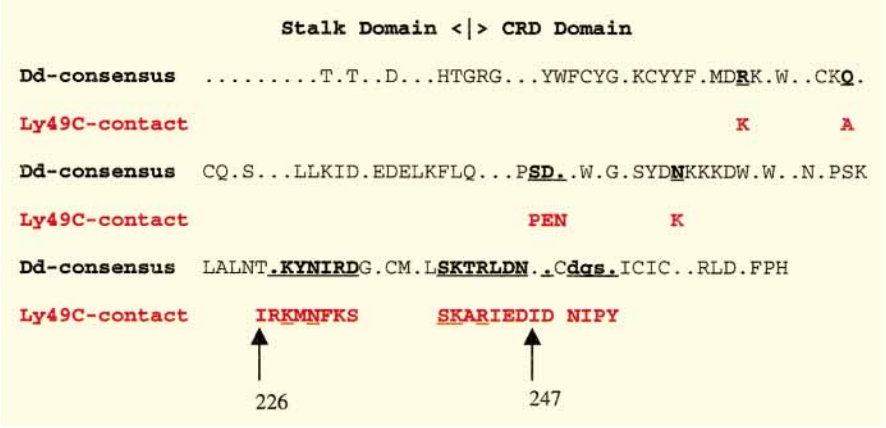

***Fig. 2.* The majority of proposed Ly49/class I contact residues are conserved in all H2-Dd-binding Ly49 proteins with the exception of Ly49C.** The consensus amino acid sequence derived from the comparison of Ly49A, D, G, O, P, R, V and W is shown. Amino acids that are found in eight out of eight sequences are shown in uppercase, while residues within the putative MHC class I contact regions that are found in six of eight receptors are shown in lowercase. Dashes represent the positions of non-conserved amino acids. Residues shown to be involved in Ly49A/class I H2-Dd binding by Tormo et al. (27) are underlined in bold type. The amino acid residues of Ly49C that correspond to the proposed contact residues of Ly49A are shown below the consensus sequence. Amino acids of Ly49C that are identical to the consensus are underlined. Arrows indicate the position of residues required for Dd binding as described by Lian et al. (28).

lowed three residues later by a hydrophobic residue (Y*xxL/V). This set the stage for the description of other receptors that contain ITIMs. It was quickly noted that both Ly49s and KIRs possess cytoplasmic ITIMs (33), and in 1996 Burshtyn et al. demonstrated that KIRs become tyrosine phosphorylated and recruit src homology 2 (SH2) domain-containing proteins and refined the consensus for an ITIM to include hydrophobic residues at the -2 position relative to the phosphotyrosine (I/VxY*xxI/L/V) (34). Olcese et al. then demonstrated the ability of phosphopeptides derived from Ly49s to interact with protein phosphatases (35). A subsequent study by our group demonstrated differential tyrosine phosphorylation of Ly49s following pharmacological stimulation. Ly49s A, C/I and G2 were efficiently tyrosine phosphorylated following pervanadate treatment. In contrast, Ly49D, a receptor that unlike others within the Ly49 family appeared to activate NK cells, was not phosphorylated (36). In addition, we were able to demonstrate the ability of phosphorylated Ly49s to co-immunoprecipitate SH2 domain-containing protein phosphatase (SHP)-1. Interestingly, even though no inhibitory Ly49 has more than one ITIM, inhibitory Ly49s contain various numbers of additional cytoplasmic tyrosine residues. Moreover, our data suggested a correlation between the number of additional tyrosines and the levels of phosphorylation we detected, suggesting that these additional residues might be phosphorylated themselves or facilitate the phosphorylation of the ITIM tyrosine residue. However, our detailed mutagenesis of the additional tyrosine residues of Ly49G2 demonstrated that these tyrosines were not phosphorylated, nor were they required for phosphorylation of the ITIM residue or the inhibitory activity of the receptor (36).

There is currently little data that addresses the mechanism leading to inhibitory Ly49 phosphorylation. Data from KIR studies have suggested that members of the src family of protein tyrosine kinases are responsible for the phosphorylation of the KIR ITIM (37). In addition, it has been shown that ITIM phosphorylation is greater when inhibitory receptors are co-engaged with activating receptor complexes (38). Together, these findings suggest a model where src family kinases activated during effector:target interactions or ligation of other activating receptors mediate the phosphorylation of the Ly49 ITIMs, resulting in the recruitment and activation of SHP-1 and the subsequent suppression of tyrosine-based signaling. Consistent with this model, Ly49s exist as homodimers where each chain provides a single ITIM, whereas KIR are monomeric but each receptor chain contains two ITIMs. The two-ITIM functional unit may be a mechanism for efficient recruitment and activation of SHP-1, which contains two SH2 domains (39, 40). Further support for this model comes from studies of Ly49A function in mice deficient in SHP-1. Ly49A-mediated inhibition is diminished in the NK cells of these mice but is not completely absent (41). The presence of residual Ly49-mediated inhibition in SHP-1-deficient animals may be due to the ability of the ITIMs of Ly49s to bind to inhibitory mediators other than SHP-1 (35).

Although the role for the Ly49 and SHP-1 is clear in NK-

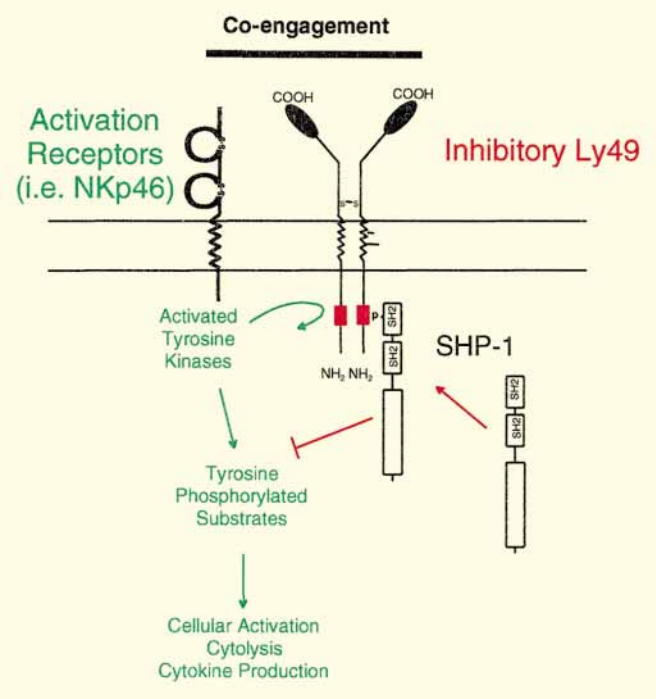

Fig. 3. Model of Ly49-mediated inhibition. See text for details.

cell inhibition, the molecular targets of SHP-1 in murine NK cells are not known. Work in the KIR system has suggested several potential targets, including the signaling chain T-cell receptor (TCR) ζ, Zap70, phospholipase Cγ (PLCγ), and the adaptors linker for activation of T cells (LAT) and SH2 domain-containing leukocyte protein (Slp)-76 (42–44). Whether these same targets are dephosphorylated by Ly49-recruited SHP-1 is presently unclear, as is the validity of these targets, because of the reported normal NK cytotoxicity in LAT or Slp-76 knockout mice (45, 46).

Taken together, the studies on Ly49-mediated inhibition are consistent with the findings of human KIR. They suggest a model where, during target cell interrogation, the presence of inhibitory Ly49 ligands results in the co-clustering of the inhibitory receptors with the activation complex (Fig. 3). The result is tyrosine phosphorylation of the ITIM of the Ly49s, likely mediated by src-family kinases activated by activation receptors such as NKp46. SHP-1 is therefore positioned in close proximity with potential substrates. This model predicts that suppression of src-family activity and/or prevention of co-clustering of Ly49s with activation complexes would prevent the phosphorylation of Ly49s and prevent them from recruiting SHP-1 or suppressing cytotoxicity. More studies into the mechanisms that lead to Ly49s are clearly needed to thoroughly test this model.

Signaling by activating Ly49s

Not all Ly49s contain cytoplasmic ITIMs. It has now become clear that Ly49s or KIR lacking ITIMs deliver activation signals to NK cells. The first clues of the biochemical mechanism of Ly49-mediated activation came from our studies of Ly49 phosphorylation. Immunoprecipitations of Ly49D, an activating receptor, under non-reducing conditions showed no receptor phosphorylation but co-immunoprecipitation of a tyrosine-phosphorylated protein with an estimated mass of 32 kDa. This co-precipitating protein reduced to phosphorylated monomers with a mass of 16 kDa, and we termed this protein pp16 (16). Our analysis demonstrated that pp16 was associated with Ly49D prior to receptor engagement and that cross-linking the receptor resulting in a prominent, transient phosphorylation of pp16. The co-immunoprecipitation of Ly49D and pp16 was consistent with the expression of both TCRζ and FcRγ in NK cells and the presence of a positively charged residue (Arg^{54}) in the Ly49D transmembrane segment. Other multichain immune recognition receptors such as the TCR, BCR and FcR, utilize a charged residue within their transmembrane domain for the interaction with signaling chains carrying the opposite charge. Pp16 co-immunoprecipitated with Ly49D in TCRζ and FcRγ double knockout mice, suggesting that pp16 was a novel signaling chain (16). In fact, we demonstrated that mutation of Arg^{54} within Ly49 ablated both pp16 association and Ly-49D-mediated signaling (16). Based on the characteristics ascribed to pp16 by our group and similar characteristics described for a small protein that co-immunoprecipitates with activating KIR (47), Lanier's group cloned a KIR-interacting protein and named it Dap12 (48). Using a murine Dap12 cDNA and anti-pp16 antisera, we were able to confirm that pp16 was Dap12, and that it binds to and mediates the signaling of the activating Ly49s D, H and P (49–51).

Dap12 is a 113 amino acid protein with a non-phosphorylated predicted mass of 12 kDa. The chain contains 12 extracellular amino acids including a CxC motif likely involved in its homodimerization. The transmembrane span includes an aspartic acid residue for interaction with Arg^{54} of Ly49D (48). Most importantly, the Dap12 cytoplasmic tail includes a single ITAM. Unlike the ITIM, an ITAM contains two tyrosine residues in configuration for phosphorylation. Both tyrosines of Dap12 are followed by hydrophobic residues at +3 relative to the phosphotyrosine. In addition, the dual tyrosines of an ITAM are precisely spaced for engagement of the dual SH2 domains of the Syk/Zap70 kinases that are both expressed in NK cells (52, 53). In fact, phosphorylated peptides derived

from Dap12 bind both Syk and Zap70 from cellular lysates (48).

Ligation of Ly49D leads to tyrosine phosphorylation of not only Dap12, but several other substrates as well (53). Therefore, using a rat NK-cell line engineered to express mouse Ly49D, we have begun the dissection of the Ly49D pathway in an effort to understand the role this protein plays in the regulation of NK-cell biology. Our initial work demonstrated that Syk, but not Zap70, was phosphorylated following Ly49D/Dap12 engagement. Moreover, we could detect an increase in Syk catalytic activity following Ly49D cross-linking, whereas Zap70 activity was unaffected. Finally, we showed that dominant negative Syk but not Zap70 was able to suppress Ly49D signaling (53). The apparent Syk dependence of Dap12 signaling is consistent with the expression of Dap12 in cells that do not express significant levels of Zap70. Interestingly, despite this biochemical evidence that Dap12 signaling is dependent on Syk, and our recent finding that pharmacological inhibitors of Syk efficiently block downstream events (54), NK cells derived from $Syk^{-/-}$ fetal liver cells appear to signal via Ly49D/Dap12 (55). The most likely explanation for these results is that development of $Syk^{-/-}$ NK cells results in a shift in their signaling to Zap70. This model could be tested via biochemical analysis of the $Syk^{-/-}$ NK cells.

The events that occur downstream of Syk activation remain poorly defined. We and others have demonstrated tyrosine phosphorylation of PLCγ, mobilization of intracellular calcium and activation of the mitogen-activated protein kinases (Mapk) extracellular signal-regulated kinase 1 and 2 (Erk1/2) (53). We have also recently demonstrated tyrosine phosphorylation of LAT (56). Consistent with both LAT and Erk1/2 activation, we recently demonstrated an increase in the binding of activator protein-1 complexes by electrophoretic mobility shift assay. Gene chip analysis suggested that Ly49D/Dap12 cross-linking leads to substantial activation of a number of genes, most notably several chemokines, and treatment with inhibitors of Mapk or Syk efficiently suppress chemokine production (54). At present, the pathways connecting Dap12 phosphorylation with Erk1/2 activation and ultimately gene transcription are completely unknown. Moreover, the ability of antibodies to Ly49 to mediate reverse antibody-dependent cellular cytotoxicity suggests that the Ly49D/Dap12 pathway can also couple to the cytolytic machinery (57). Natural cytotoxicity is dependent on phosphoinositide-3 kinase (PI3K), Erk1/2 and the stress-activated Mapk p38 (58–61). Inhibition of any of these pathways inhibits cytolytic activity, suggesting that Ly49D may also activate p38 and/or PI3K. Notably, however, treatment of NK cells with inhibitors of p38 does not block Ly49D-mediated production of cytokines or chemokines (54). These issues are still currently under investigation.

Based on these studies, we can begin to formulate a model to explain Ly49-mediated NK activation (*Fig.* 4). During target interrogation, the presence of ligands for activating Ly49s leads to the co-engagement of Ly49s and the NK cytolytic activation complex comprised of NKp46 or other target recognition receptors. The activating Ly49s likely mediate the phosphorylation of Dap12 via the Src-family kinases. Once phosphorylated, Dap12 recruits and activates the Syk/Zap70 kinase Syk. Our studies suggest that Syk then mediates all the downstream events. We propose that the Syk-mediated phosphorylation of Cbl is involved in the regulation of the Dap12 complex, and that the activation of Mapk by Ly49D is required for the transcriptional events associated with engagement. Through the production of cytokines and chemokines, we propose that activating Ly49s participate not only in augmenting cytolytic signals initiated by other receptors but also in the recruitment of other effector cells to an immune site. A principal role for Dap12 in "calling out the troops" is supported by the potent induction of chemokines we have documented and in the defects in immune priming evident in the Dap12 knockout and loss-of-function mice.

As we have investigated the Ly49 repertoire of various strains of mice, we have documented what may represent diverse Ly49/Dap12 signaling capabilities as well. Initial analysis of Dap12-mediated signal transduction in a variety of gene-targeted mice suggested that Dap12 signaling is impaire din mice of the 129/J background. Cross-linking of Ly49D induced Dap12 phosporylation but failed to cause calcium mobilizationin 129/J NK cells, and these same cells failed to demonstrate Ly49D-mediated lysis of Chinese hamster ovary targets. Each of these deficits was seen despite the detection of activation-induced Dap12 phosphorylation. As mentioned above, we have cloned the Ly49D-like activation receptor of 129/J mice and named it Ly49R (18). The sequence of Ly49R suggests no basis for its lack of function in 129/J NK cells. In fact, when the Ly49D/Dap12 receptor complex is reconstituted in HEK293 T cells, Ly49R signals just as well as Ly49D. In addition, transfection of Ly49R or D into a human $Dap12^{+}$ cell line also demonstrates similar function for the two genes. Furthermore, we have demonstrated by cDNA cloning that 129/J-derived Dap12 is identical to that of C57BL/6 mice. Together, these findings suggest a signaling deficit in the 129/J NK cells themselves. We have documented defects in the 129/J Dap12 signaling pathway by expressing Ly49D on NK cells sorted from 129/J or C57BL/6 mice. After infection

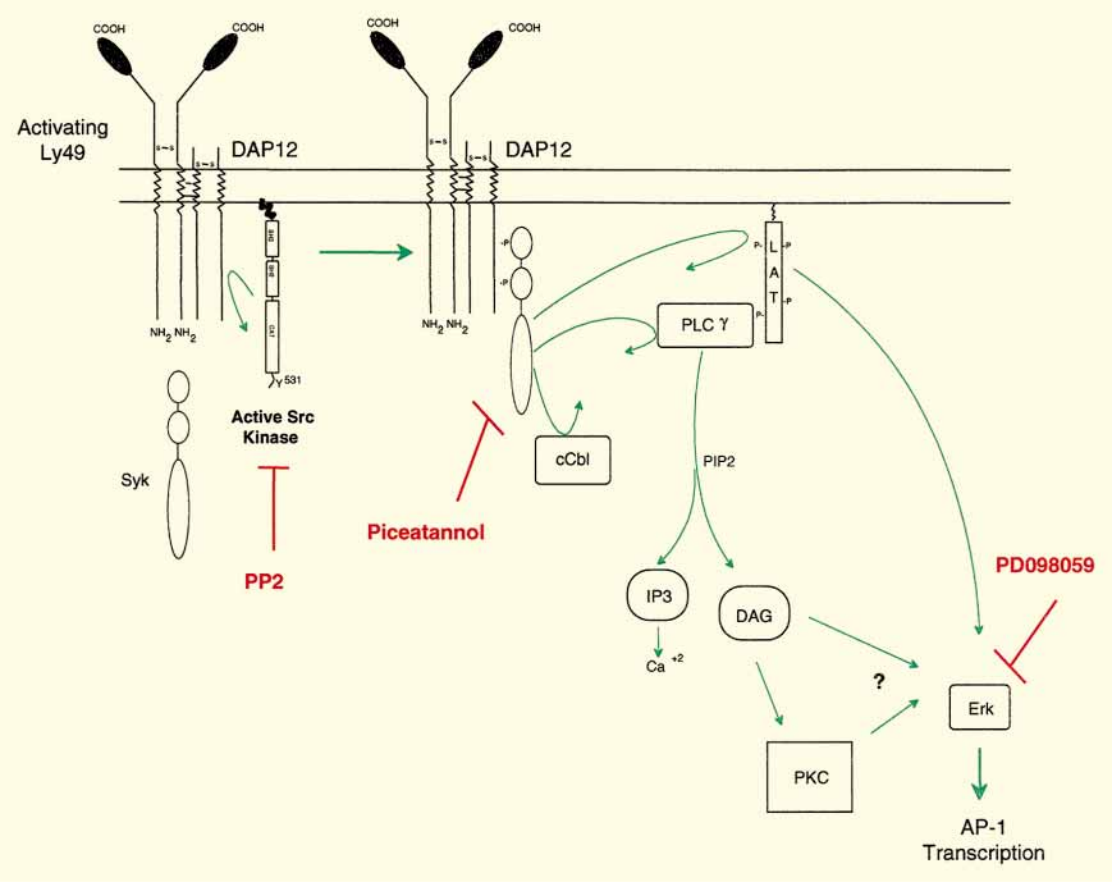

Fig. 4. Pathways involved in the activation of NK cells via Ly49D/Dap12. Mediators include phosphatidylinositol 4,5-bisphosphate (PIP2), inositol 1,4,5-trisphosphate (IP3), diacylglycerol (DAG), phospholipase Cγ (PLCγ), the linker for activation of T cells (LAT), extracellular signal regulated kinase (Erk) and protein kinase C (PKC). Compounds in bold inhibit the signal transduction cascade at the points indicated. See text for details.

with a vaccinia construct, the C57BL/6 NK cells expressed ample Ly49D and mobilized intracellular calcium upon cross-linking. In contrast, cross-linking had no effect on the 129/J NK cells even though they expressed Ly49D. These experiments provide strong evidence that the Ly49/Dap12 signal transduction cascade is defective in 129/J mice even though calcium mobilization induced by the TCR of these mice is normal. These findings are particularly important due to the common use of 129 substrains in the generation of gene-targeted mice and the reported differences in the immune responses between these strains. For example, due to the genetic background of their immune cells, 129 mice are highly susceptible to infection with Sendai virus (62). In addition, 129 mice are highly susceptible to the induction of experimental systemic lupus erythematosis (63), and there are marked differences in the susceptibility of C57BL/6 and 129 mice to EAE (64). 129/J mice are also highly susceptible to murine cytomegalovirus (65), and the antiviral response of resistant strains is mediated by NK cells (66, 67). Moreover, analysis of bone marrow graft rejection, a process regulated by Ly49-bearing cells, showed that 129/J mice failed to reject, or weakly rejected, marrow from a variety of donor strains that were rejected by C57BL/6 mice (68). All of these differences are observed despite the fact that C57BL/6 and 129/J mice share the same MHC haplotype, H-2^b. Our findings suggest that some of the immunological characteristics attributed to 129/J mice, and/or to gene-targeted animals derived from this strain, may be due to an apparent blockade in Dap12 signaling somewhere between Dap12 phosphorylation and calcium mobilization.

The significance of the continued study of the signal transduction cascade of Dap12 was recently underscored by the report of the immune phenotype of mice deficient in Dap12 or Dap12 signaling (69, 70). These mice show a pronounced deficit in the ability to respond to immunological challenge. They have increased numbers of cutaneous dendritic cells (a

cell population that also expresses Dap12), they have a reduced response in a model of EAE (69), and they have a pronounced reduction in contact hypersensitivity (70). These data suggest that understanding the signaling mechanisms involved in the Ly49/Dap12 response could lead to therapeutic targets for the treatment for a variety of immune disorders.

In summary, our genetic and biochemical analysis of both the Ly49 repertoire and Ly49 signaling has unveiled an increasingly complex picture of the class I binding receptors of murine NK cells. The results suggest extreme caution is warranted when using receptor-specific reagents outside of the strain in which they were originally characterized. The unique repertoire of 129/J mice further suggests that mice may also possess a large number of unique receptor populations that may significantly impact the immune status of those strains. To further complicate matters, our description of a signaling deficit in 129/J mice suggests that immunological phenotypes previously attributed to gene-targeted mice, or the 129/J strain specifically, may be the result of aberrant Dap12 signaling. Regardless, together with the continued study of the complex repertoires of the KIR, continued study of the murine Ly49s should shed light on the underlying role of these large receptor families in the control of the immune response.

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